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Short communication

Production of *ortho*-hydroxydaidzein derivatives by a recombinant strain of *Pichia pastoris* harboring a cytochrome P450 fusion gene

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ABSTRACT

CYP57B3 from *Aspergillus oryzae* was recently discovered to catalyze the *ortho*-hydroxylation of the soyisoflavone genistein. In the present study, the gene encoding CYP57B3 was fused with the reductase domain of the CYP102A1 gene (BM3R) from *Bacillus megaterium*, and recombinant *Pichia pastoris* harboring the P450 fusion gene was evaluated for its ability to produce *ortho*-hydroxydaidzein derivatives from daidzein. The results showed that 8-hydroxydaidzein (8-OHDe), 3′-hydroxydaidzein (3′-OHDe), and 6-hydroxydaidzein (6-OHDe) were produced during fermentation with a maximal conversion of 2.4, 0.9, and 36.3%, respectively. The maximal yield of 6-OHDe by the recombinant strain was 9.1 mg/l. To our knowledge, both the maximal yield and the conversion efficiency of 6-OHDe from daidzein in the present study are the highest among those reported in the literatures to date. The present study is also the first to demonstrate production of *ortho*-hydroxydaidzein derivatives using a fusion fungus cytochrome P450 enzyme.

1. Introduction

Daidzein, one of the major isoflavones found in soybeans [1], has been under intensive investigation due to its potential role in preventing certain hormone-dependent maladies and other diseases [2]. In recent years, *ortho*-hydroxydaidzein derivatives, including 8-OHDe, 6-OHDe, and 3′-OHDe (Fig. 1), have attracted much attention because of their pharmaceutical activities, including anti-cellular proliferation [3], aldose reductase inhibition [4], anti-mutagenesis [5] and enhanced cancer chemotherapeutic activity [6]. In addition, during our continuing search for natural tyrosinase inhibitors for use in skin-whitening cosmetics, we demonstrated that 6-OHDe [7] and 8-OHDe [8,9] are potent tyrosinase inhibitors and also demonstrated the skin depigmenting activity of 8-OHDe in clinical trials with human volunteers [10]. Due to their many bioactivities, the production of *ortho*-hydroxydaidzein derivatives has become a subject of great interest.

Ortho-hydroxydaidzein derivatives were originally discovered in soybean products fermented by *Aspergillus* [11] and *Micrococcus* [12]. During production of these foods, daidzein was transformed into *ortho*-hydroxydaidzein derivatives by cytochrome P450 monooxygenases (CYP), a super-family of heme-containing enzymes (Fig. 1) [13]. During the catalysis process, P450 must be associated with electron donor partner proteins (cytochrome P450 reductase, CPR) to transfer two electrons from NAD(P)H to the P450 heme domain. However, the electron transfer between CPR and P450 usually limits the reaction rate of P450s. Another kind of P450s is self-sufficient. For example, CYP102A1 (BM3) from *B. megaterium* is encoded in a single polypeptide that possesses a CPR domain fused to the heme domain [14]. Self-sufficient P450s usually exhibit great catalytic activity due to the high efficiency of the electron transfer from CPR to its fused P450 partner. Recently, some studies mimicked the natural self-sufficient P450s and developed artificial self-sufficient P450s by genetic fusion of non-self-sufficient P450 with CPR. As a result, the catalytic activities of those artificial self-sufficient P450s were greatly improved [15].

A. oryzae is the best-known microorganism that transforms soyisoflavones into their corresponding *ortho*-hydroxyl derivatives. This microorganism contains 155 putative P450 genes in its genome, from which 142 P450 proteins are expressed [16]. Among them, CYP57B3 was recently shown to catalyze *ortho*-hydroxylation of the soyisoflavone genistein in cooperation with a CPR from *Saccharomyces cerevisiae* [17]. In the present study, CYP57B3 was fused with BM3R from *B. megaterium* to form an artificial, self-sufficient P450, and the production of *ortho*-hydroxydaidzein derivatives from daidzein by recombinant *Pichia pastoris* harboring the fusion gene were evaluated.

2. Materials and methods

2.1. Microorganisms and chemicals

A. oryzae (BCRC32288) and *B. megaterium* (ATCC14581) were obtained from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) and cultivated according to the BCRC protocol. The EazySelect™ Pichia expression kit, containing *P. pastoris* X-33, plasmid

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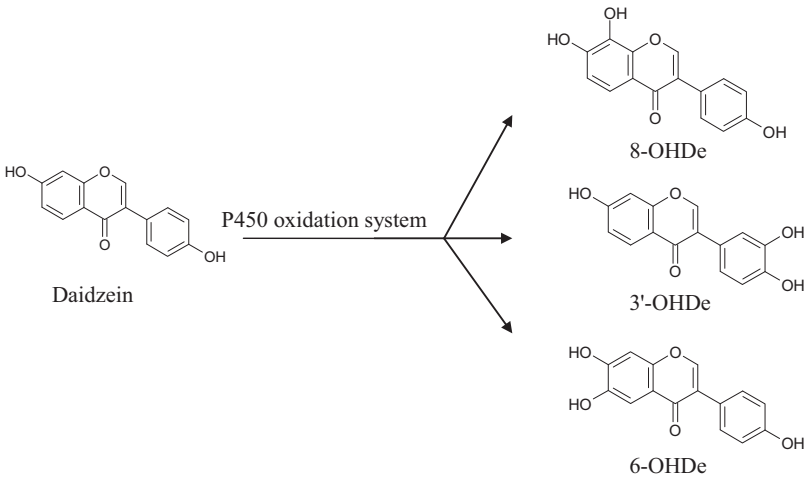

Fig. 1. Biotransformation of daidzein to *ortho*-hydroxydaidzein derivatives by P450 oxidation systems.

vector pGAPZA™, and the antibiotic Zeocin™ were obtained from the Invitrogen Company (Carlsbad, CA). Daidzein and 6-OHDe were purchased from Sigma–Aldrich (St. Louis, MO). 8-OHDe was kindly donated by Dr. Hai-Liang Zhu at the Institute of Functional Biomolecules of Nanjing University, Nanjing, PR China. 3′-OHDe was obtained from Alfa Aesar (Ward Hill, MA). Other reagents and solvents used were commercially available and were used as received.

2.2. Construction and expression of the CYP57B3-BM3R fusion P450 in P. pastoris

The CYP57B3 gene was PCR amplified from cDNA from *A. oryzae* (BCRC32288), using specific sense (ccgaattcaaaatgatagggacggtcttggacaca) and antisense (tctagatctagccacattttcgaccggcttccg) primers. BM3R was PCR amplified from the genomic DNA of *B. megaterium* (ATCC14581) using specific sense (cccagatctcctcgcaaaaaggcagaaaacgctc) and antisense (aaactcgagccagcccacacgtcttttgcgtat) primers. The amplified CYP57B3 and BM3R genes were sequenced and cloned into the EcoRI-BglII and BglII-XhoI sites, respectively, of a pETDuet-1™ vector (Invitrogen) to form the fusion gene. Then, the fusion gene was cut by the restriction enzymes EcoRI and XhoI and cloned into the corresponding sites of the pGAPZA vector to form pGAP-CYPBM3R.

The resulting vector was then linearized with the DraI restriction enzyme and transformed into *P. pastoris* X-33 using an electroporation method from the expression kit manual. The recombinants were selected with Zeocin, and the insertion of the fusion gene into the genomic DNA of the recombinants was confirmed using the PCR method described above.

2.3. Fermentation and HPLC analysis of ortho-hydroxydaidzein derivatives

Recombinant *P. pastoris* were cultivated in 20 mL of yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose), containing 100 μg/ml of Zeocin, 250 μM of δ-aminolevulinic acid, and 100 μM of daidzein, with 240 rpm shaking at 30 °C. At the indicated time intervals, cells were harvested, extracted with MeOH/ACN (50%:50%), and analyzed by HPLC. The operational conditions for the HPLC analysis using an analytic C18 reversed-phase column (Spherisorb, 5 μm, 4.6 i.d. × 250 mm, ODS 2, Phase Separation, Deeside Industrial Park, Clwyd, UK) include a gradient elution using water (A) containing 1.5% (v/v) acetic acid and acetonitrile (B) with an initial isocratic elution for 4 min with 15% B, followed by a linear gradient for 20 min with 15% to 35% B, a final isocratic elution for 10 min with 35% B at a flow rate of 1 ml/min, and detection of the absorbance at 254 nm. The identities of the resulting *ortho*-hydroxydaidzein derivatives were confirmed by comparison with the observed retention times of the standards. The quantities of the resulting *ortho*-hydroxydaidzein derivatives were calculated using standard curves obtained from the HPLC analysis of the standards.

3. Results and discussion

Nazir et al. first discovered that CYP57B3 could catalyze the *ortho*-hydroxylation of both isoflavone genistein and flavanone naringenin *via* cooperation with *S. cerevisiae* CPR [17]. However, they did not mention catalytic activity of CYP57B3 toward the isoflavone daidzein. Due to the many activities of *ortho*-hydroxydaidzein derivatives [3–6], and in particular, their skin-whitening capabilities [7–10], we investigated the transformation of daidzein by CYP57B3 in the present study. We used an EazySelect™ Pichia expression system (Invitrogen) for this study because *P. pastoris* is potentially useful for industrial applications as well as basic research and the yeast itself cannot transform daidzein to the various *ortho*-hydroxydaidzein derivatives (data not shown). To remove the rate-limiting step observed in most P450 enzyme reactions, we fused CYP57B3 with the RM3R. Fig. 2 shows a map of the complete plasmid, in which a linker sequence containing a BglII restriction site and an additional proline codon (cct) joins CYP57B3 and BM3R.

The recombinant *P. pastoris* harboring the CYP57B3-BM3R fusion gene was cultivated in the presence of 100 μM of daidzein, and the resulting *ortho*-hydroxydaidzein derivatives from the fermentation broth were analyzed by HPLC. Fig. 3 shows the HPLC profile of the fermentation broth after 96-h of incubation. These results show that at least three *ortho*-hydroxydaidzein derivatives, 8-OHDe, 3′-OHDe, and 6-OHDe, with HPLC retention times of 18.2, 22.0, and 23.2 min, respectively, were produced during fermentation. Two unknown metabolites, with HPLC retention times of 24.0 and 24.6 min, also appeared during fermentation. The production of the three *ortho*-hydroxydaidzein derivatives increased

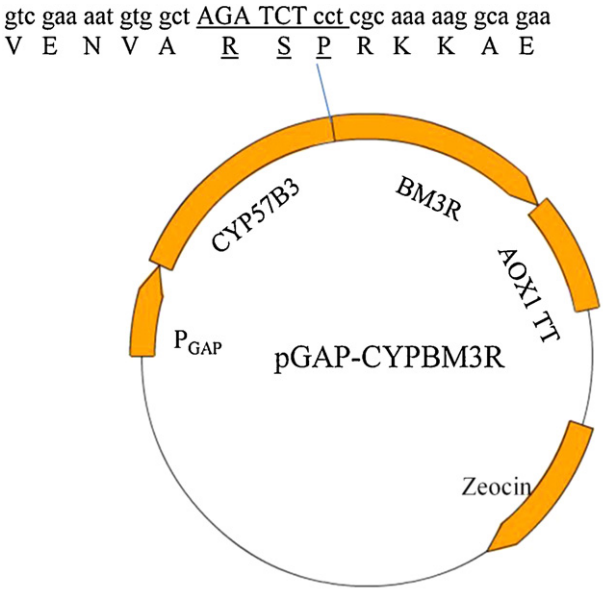

Fig. 2. The map of the constructed expression vector pGAP-CYPBM3R. The linker sequence joining both CYP57B3 and BM3R is shown underlined.

Table 1
Ortho-hydroxydaidzein production by recombinant *E. coli* or *P. pastoris* harboring heterogeneous P450 enzymes.

P450 name	P450 sources	Recombinant Hosts	Feeding daidzein (μM)	Conversion (%) 8-OHDe	6-OHDe	3′-OHDe	Reference
nfa33880[a]	*N. farcinica* IFM10152	*E. coli*	100	2.7	2.0	–	[18]
CYP107H1[a]	*B. subtilis* 168	*E. coli*	50	–[e]	–	0.6	[19]
rCYP105D7[b]	*S. avermitilis* MA4680	*E. coli*	100	–	–	2.1	[20]
CYP102D1[c]	*S. avermitilis* MA4680	*E. coli*	100	–	–	–	[21]
mCYP102D1[d]	*S. avermitilis* MA4680	*E. coli*	100	4.2	8.8	–	[21]
rCYP57B3[b]	*A. oryzae* BCRC32288	*P. pastoris*	100	2.4	36.3	0.9	This study

[a] Co-expressed with ferredoxin reductase (camA) and ferredoxin (camB).
[b] Engineered fusion P450.
[c] Wild-type self-sufficient P450.
[d] F96V/M246I mutated self-sufficient P450.
[e] Not detected.

with time from 24 h to 96 h of cultivation, while an opposite trend of remaining daidzein substrate revealed (Fig. 4). In the recombinant *P. pastoris*, the pGAP-CYPBM3R expression plasmid containing the expression cassette driven by a constitutive glyceraldehyde-3-phosphate dehydrogenase gene promoter is integrated into the host chromosomal DNA to form a stable expression system. Therefore, the production of *ortho*-hydroxydaidzein derivatives was directly correlated with the growth of recombinant *P. pastoris*. The maximal yields of 8-OHDe, 3′-OHDe, and 6-OHDe were 0.58, 0.23, and 9.1 mg/l, respectively. These results revealed that 6-OHDe is the dominant product for the transformation of daidzein by the CYP57B3-BM3R fusion P450. Nazir et al. has showed that non-fusion CYP57B3 cooperation with *S. cerevisiae* CPR selectively produced 3′-hydroxyl derivative of genistein [17]. *A. oryzae* itself, where CYP57B3 cooperates with intact *A. oryzae* CPR, selectively produced 8-hydroxyl derivatives from both daidzein and genistein [11]. Our results differ from these previous studies. It is possible that "cooperation with a different CPR" or "fusion action" affected the catalytic position of the substrate in the fusion protein. The results from Choi et al. support the first hypothesis. They used different CPRs to change the catalytic properties of P450 [18], but the detailed mechanism needs to be resolved further.

Kim et al. have thoroughly studied the production of *ortho*-hydroxydaidzein by soil bacteria, including *Bacillus subtilis*, *Nocardia farcinica*, and *Streptomyces avermitilis* [18–21]. They identified P450 enzymes with daidzein hydroxylase activity from bacteria and expressed these P450s in *Escherichia coli* to produce *ortho*-hydroxydaidzein derivatives. The conversion of *ortho*-hydroxydaidzein derivatives by recombinant *E. coli* in these previous studies is compared with the present study and summarized in Table 1. As shown in this table, the conversion of 6-OHDe from daidzein in the present study is the highest. To the best of our knowledge, the yield of 6-OHDe here is also the highest reported in the literature. Because 6-OHDe is a potent tyrosinase inhibitor and useful for various cosmetic purposes [7], recombinant *P. pastoris* in the present study has great potential for the industrial production of 6-OHDe.

CYP57B3 from the fungus *A. oryzae* produces three *ortho*-hydroxyl isoflavone derivatives in both recombinant *S. cerevisiae* [17] and *P. pastoris* (this study), while daidzein hydroxylase P450s from the various soil bacteria studied can produce only one (3′-OHDe) or two (6-OHDe and 8-OHDe) *ortho*-hydroxydaidzein derivatives in recombinant *E. coli* (Table 1). In addition, CYP57B3 has been shown to catalyze the conversion of the isoflavone genistein, the flavanone naringenin [17], and the isoflavone daidzein (this study). However, the flavonoid hydroxylase P450s from the soil bacteria studied were proven to recognize only one flavonoid as a substrate [22]. These results reveal that CYP57B3 has greater flexibility in both binding substrates and catalytic positions than those of the P450s from known soil bacteria sources. Hence, examining whether CYP57B3 could catalyze the conversion of other

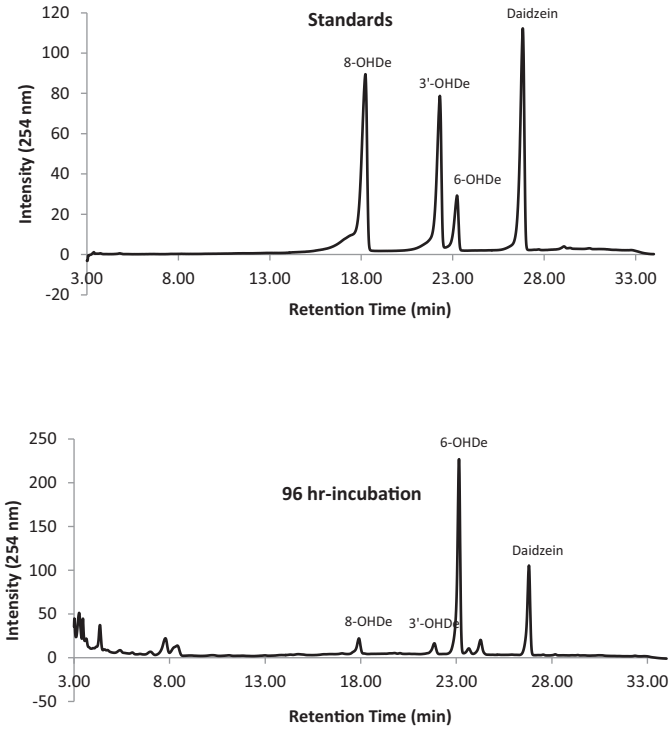

Fig. 3. HPLC profile of production of *ortho*-hydroxydaidzein derivatives by recombinant *P. pastoris* harboring pGAP-CYPBM3R.

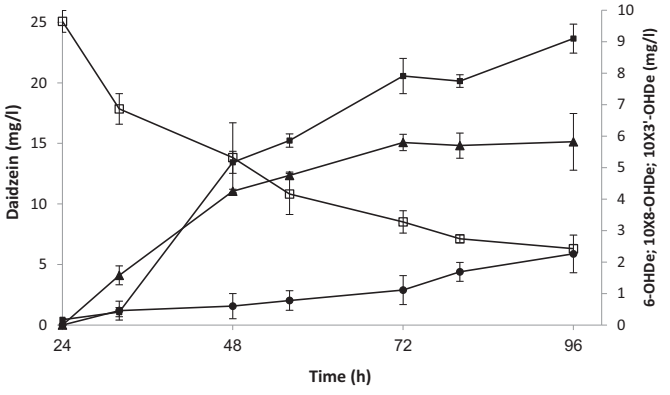

Fig. 4. Time course of consumption of daidzein (□) and production of *ortho*-hydroxydaidzein derivatives, including 8-OHDe (▲), 3′-OHDe (●), 6-OHDe (■) by the recombinant *P. pastoris*. The average ($n = 3$) is presented with the S.D. represented by error bars.

flavonoids, and even engineering or mutating the enzyme to produce new hydroxyl flavonoids, are interesting issues and worth further study.

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